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Multiple Reaction Monitoring Mass Spectrometry is a powerful tool to study glycerolipid composition in plants with different level of desaturase activity

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Abbreviations: MGDG, Monogalactosyldiacylglycerol; DGDG, Digalactosyldiacylglycerol; MS, mass spectrometry; MRM, Multiple Reaction Monitoring; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol

In plants, two lipid desaturation pathways exist. A so-called prokaryotic pathway is active in plastids and responsible for unsaturation of 16 carbon fatty acids. An eukaryotic one, in the endoplasmic reticulum, acts on 18 carbon fatty acids. Desaturase activities are affected in stressed plants, and conversely, they have an impact on the capability of plants to adapt to stress. So knowing lipid unsaturation is important for physiological studies. Analysis of lipids by mass spectrometry, in the multiple reaction mode, gives access to the molecular species present in each membrane lipid class. We illustrate the powerfulness of this technique by applying it to phospholipids and galactolipids extracted from plants where the desaturation pathways are present at variable level.

The synthesis of glycerolipid (i.e., phospholipids, galactolipids and triacylglycerides) in plant is dependent on two distinct pathways.[1] Fatty acid synthesis begins in the chloroplast and is either continued by the prokaryotic plastidial pathway of the envelope, or, after export, by the eukaryotic pathway present in the endoplasmic reticulum.[2] The plastidial pathway generates phosphatidic acid, where *sn*-2 position of glycerol is occupied by a 16-carbon (C16) fatty acid. This lipid is at the origin of the synthesis of phosphatidylglycerol (PG) or of prokaryotic galactolipids. Because of specificity of acyltransferases[3] and successive desaturations, these prokaryotic galactolipids are mainly esterified by a linolenate (18:3) fatty acid at the *sn*-1 position and a hexadecatrienoic (16:3) fatty acid at the *sn*-2 position. On the contrary, the eukaryotic pathway synthesizes lipids with 16- or 18-carbon (C16 or C18) fatty acids at *sn*-1 position and only C18 fatty acids at the *sn*-2 position. Phosphatidylcholine (PC) synthesized through this latter pathway can be transferred to the chloroplast where it will be at the origin of eukaryotic galactolipids (C16 fatty acids are esterified at the *sn*-1 position, exclusively).

In some plants, both pathways are used to synthesize galactolipids,[4] that contain high amounts of 16:3 and 18:3 fatty acids. These plants are referred to as 16:3 plants. In other plants, galactolipid synthesis is only dependent on the eukaryotic pathway, so they do not contain 16:3 fatty acid. These plants are called 18:3 plants. Thus, the molecular species that can be found in mono- and di-galactosyl-diacylglycerides (MGDG and DGDG, respectively) of the two categories of plants will differ with respect to the presence of 16:3 fatty acids.

Fatty acid unsaturation can also be affected in the eukaryotic pathway, resulting in altered content of the different C18 fatty acids. Mutants of the different desaturases have been described in *Arabidopsis thaliana*.[5] The first desaturase is the stearate desaturase, making oleate (18:1) from stearate (18:0). In the *ssi2* mutant, this activity is reduced by 90%, resulting in an enrichment of galactolipids and phospholipids in 18:0 fatty acid.[6]

Physiologically, desaturase activities, and hence the unsaturation degree of membrane lipids, are affected during stress responses.[7-10] In fact, a change in lipid unsaturation is one aspect of plant adaptative responses, and it is required for plant survival.[11,12] Being able to detect unsaturation changes can therefore be of high importance for screening adaptative capacity of different plant species or species cultivars.

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Glycerolipid composition can be analyzed by various techniques. The first analyses relied on lipid separation by thin layer chromatography, followed by preparation of fatty acid methylesters that can be identified by gas chromatography.[13] This technique will only indicate the proportion of each fatty acid present in each glycerolipid class (e.g., MGDG, DGDG, PG, PC…) but will not indicate what the couples of fatty acids are. The development of mass spectrometry (MS)-based lipidomics methods now makes it possible to analyze the wide range of molecular species that constitute lipids more readily. Frequently, it is reported as a mass scan.[14-16] In that case, it is the mass of the lipids that is known. Yet, different molecules of glycerolipids, even with a single type of polar head, can correspond to a given mass. For instance a 16:3/18:0-MGDG will have the same mass as a 16:1/18:2- or a 16:0/18:3-MGDG; they are all 34:3-MGDG. Yet, the multiple reaction monitoring (MRM) mass spectrometry is an easy way to analyze glycerolipid that gives access to the fatty acids esterified on the glycerol backbone, and thus gives a list of the molecular species. This technique needs the tandem mass spectrometry to be implemented with collision-induced dissociation (CID). The parent glycerolipid is detected in the first quadrupole (Q1) of the tandem MS, the second quadrupole (Q2) is the intermediary stage filled with inert gas where CID of the parent glycerolipid occurs, and the resultant fatty acid ion-fragments are detected in the third quadrupole (Q3). In our examples the 16:3/18:0-MGDG ion mass will be detected in the Q1 and the 16:3 or 18:0 ion mass in the Q3. Only one type of 34:3-MGDG type will liberate 16:0 in Q3, that is 16:0/18:3-MGDG. An important point is that in this technique the *m/z* that have to be detected in Q1 and Q3 have to be pre set. A prerequisite for this technique is to know which molecular species are expected. It is an a priori technique and a preliminary work of mass scan for glycerolipid has to be previously performed.

We wanted to illustrate how powerful the MRM mass spectrometry analysis is and how useful for plant physiology studies. For this purpose, we analyzed galactolipid and phospholipid composition, on the basis of the molecular species that exist either in 16:3 and 18.3 plants or in *ssi2* mutant defective in stearate desaturase. Lipids were extracted, after grinding of leaves in hot methanol by an Ultra-Turrax homogeneizer, following a protocole adapted from Rainteau et al.[17] The HPLC separation of phosphoglycerolipid classes and galactolipid classes was performed using an Agilent 1100 HPLC system equipped with a 250 mm × 4 mm (length × internal diameter) 5 μm Lichrospher silica column. The mobile phases consisted of hexane/isopropanol/water (628:348:24, v/v) supplemented with 10 mg/L ammonium formiate and isopropanol/water (850:146, v/v) supplemented with 10 mg/L ammonium formiate. The gradient is the same as in Rainteau et al.[17] In these conditions, lipids were separated according to their polar head group. So, it was possible to define a time-window specific for each lipid class. Typically the time-windows for PC, PE, PI, PG, MGDG and DGDG were *ca.* [33–38] min, [10–24] min, [25–29] min, [12–17] min, [5–13] min and [16–27] min, respectively. Eluted lipids were continuously injected to tandem mass spectrometer (QTrap2000, ABSciex) and the MRM analysis was performed and acquired during the entire HPLC run (60 min). For further analyses, only data acquired during each lipid-specific time-window were considered. The couples of masses looked for in Q1 and Q3 were based on mass scan using specific precursor of mode or neutral loss mode (data not shown). The tables of transitions (couple of masses) analyzed for galacto- and phosphoglycero-lipids are in **Table S1.**

Figure 1 displays a representative MRM experiment for galactolipid. The HPLC with polar column does separate glycerolipids according to their head group (**Fig. 1A**). Each peak is the result of the addition of the signals for all MRM transitions analyzed in the run (**Fig. 1B and C**). For the time window corresponding to the elution of the glycerolipid of interest a MRM spectrum, consisting in the integration of all MRM peaks, can be calculated (**Fig. 1D**). Once a signal associated with each transition, it is necessary to take into account the isotopic distribution of C in lipid molecules. We considered ^{13}C to occur at 1.1% of the frequency of ^{12}C. For each MRM transition, we calculated a correction coefficient considering that the monitored transition is in part due to an isotopic overlap (Rainteau et al).[17] The correction factor was calculated for all MRM transitions, for galactolipids and phosphoglycerolipids, from the lowest to highest molecular weights. The Excel macro developed is given in **Table S1**. From the corrected MRM spectra it is possible to draw the composition in molecular species. For a glycerolipid esterified with two different fatty acids (e.g., 16:3/18:0-MGDG), then the 2 corresponding transition signals are added, while for glycerolipid esterified with one fatty acid (e.g., 18:3/18:3-MGDG) only one MRM transition reflects this molecule abundance.

Using this technique, galactolipid molecular species were determined in so-called 16:3 and 18:3 plants (**Fig. 2**). As expected, only MGDG extracted from spinach or Arabidopsis (two 16:3 plants) contains high amounts of 16:3 fatty acid (**Fig. 2A**). Molecular species in which this fatty acid is present represent between 40% (spinach) and 60% (*Arabidopsis*) of the total. Indeed, if 18:3/16:3 is the main of these molecular species, 16:3 is also found associated with 18:1 and 18:2. The other molecular species present in significant amounts are 18:2/18:3 and 18:3/18:3. These latter species represent more than 90% of the molecular species in MGDG from 18:3 plants (leek and mint). This difference in capability to desaturate C16 fatty acids is also reflected in DGDG composition (**Fig. 2B**). Although less abundant (ca. 10%), the 16:3/18:3 species is solely present in lipids from the 16:3 plants. It has to be noted that, among the other species, the 16:0/18:3 one is more present in DGDG (10 to 18%) than in MGDG (< 1.5%).

Expressed as percentage of each fatty acid vs. total fatty acids present in the chloroplastic lipids, our data are in accordance with those reported for spinach chloroplast envelope[13] or for field mint galactolipids[18] after gas chromatography analysis. However, the MRM mass spectrometry gives access to each individual molecular species, thus allowing one to detail the partners of each fatty acid, especially for the species that are represented at a low level (such as 18:1 or 18:2/16:3).

Another example of MRM-MS/MS interest comes from the analysis of *ssi2* plants, with a very low 18:0 desaturase activity

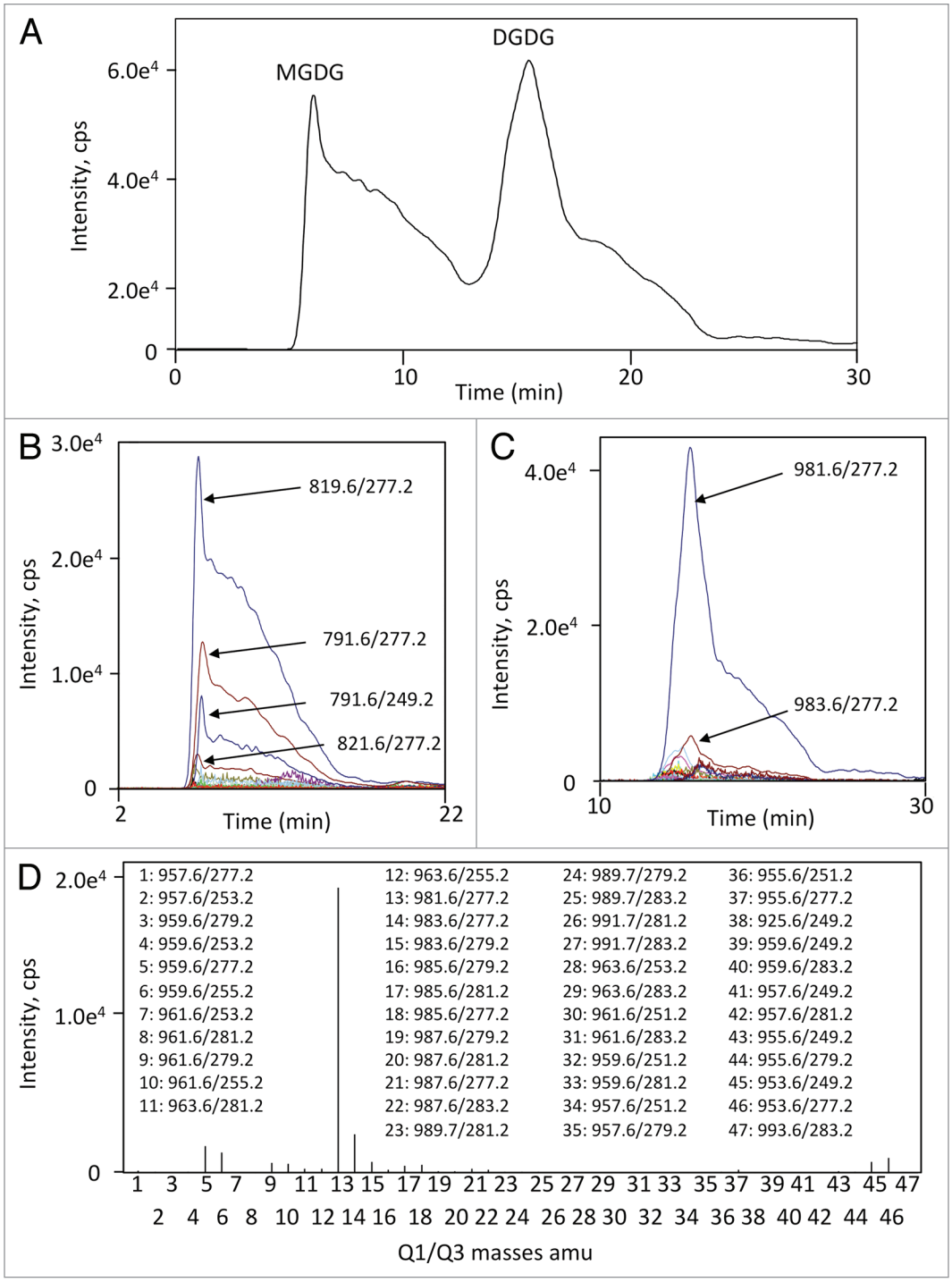

Figure 1. Representative MRM experiment. Galactolipids from a bulk lipid extract of Spinach leaves were analyzed by MRM. (**A**) Sum of the signals of all MRM transitions analyzed. The peaks corresponding to the elution of MGDG and DGDG are displayed. Each MRM transition for one galactolipid class can be visualized separately, as seen for MGDG (**B**) and DGDG (**C**). (**D**) Signal intensities are associated with each MRM transition for one lipid class, leading to the MRM spectrum for this lipid. DGDG spectrum is displayed. This spectrum is calculated within the time period corresponding to DGDG elution, therefore the data are not contaminated by isobars that would elute at different time period. For MGDG and DGDG the parent ions m/z that are analyzed in Q1 correspond to the $[M+HCOO]^-$. The ions analyzed in Q3 correspond to fatty acid.

(approx. 10% of the level in the wild type counterpart[6]). All 18:0-molecular species are enriched in the mutant MGDG (**Fig. 3A**) and DGDG (**Fig. 3B**) compared with wild type (WT), whatever the other fatty acid of the molecular species. However, the 18:0 fatty acid is preferentially associated to 18:3 one, and also to 18:2 one. In the *ssi2* mutant, these molecular species account for ca. 4% and 15% in MGDG and DGDG, respectively, instead of ca. 1% and 1.5% in WT. This increase is

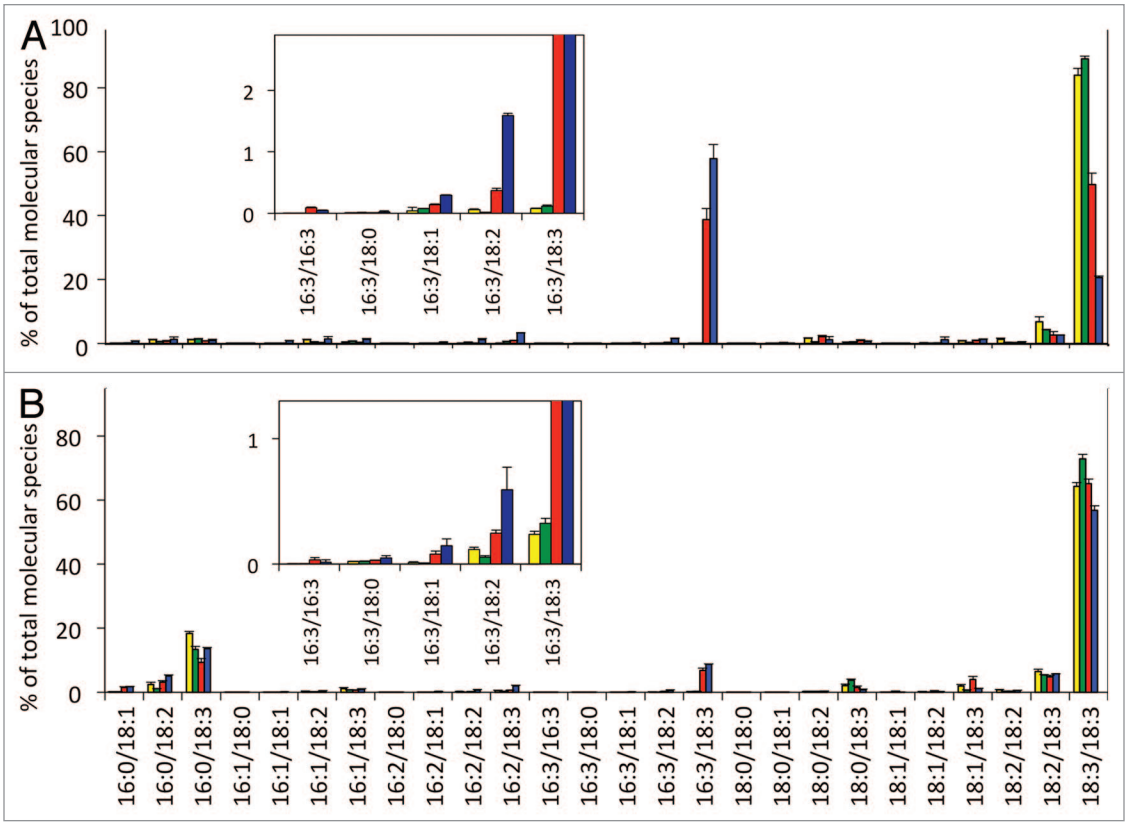

Figure 2. Molecular species composition of MGDG (**A**) and DGDG (**B**) extracted from 16:3 or 18:3 plant leaves. 16:3 plants are Spinach (*Spinacia oleracea* L., red bars) and *Arabidopsis thaliana* (cv Columbia, blue bars). 18:3 plants are Leek (*Allium porrum* L., yellow bars) and Mint (*Mentha arvensis* L., green bars). Values are means of 3 independent experiments. Insert graphs give details of 16:3-containing molecular species.

mainly compensated by a decrease in 16:3/18:3 for MGDG or in 18:3/18:3 for DGDG. These data are in accordance with those of Nandi et al.[16] who reported composition of *ssi2* lipids expressed in exact mass. However, the same mass can correspond to two, or more, species. Our data indicate that the increase in 36:2 and 36:3 galactolipids corresponds to an increase of the 18:0/18:2 and 18:0/18:3 species, not of the 18:1-containing ones.

The 18:0 fatty acid is even more present in the phospholipids of the mutant, almost exclusively in the 18:0/18:2 and 18:0/18:3 species, in agreement with Nandi et al.[16] who found an increase in 36:2 and 36:3 phospholipids. In PC (**Fig. 4A**), these species become the prominent ones as almost all those containing two unsaturated fatty acids (to the exception of 18:3/18:3) are less abundant than in WT. In phosphatidylethanolamine (PE, **Fig. 4B**) the two above species are also accumulated in the mutant. However, in contrast with PC, species with two unsaturated chains are much less reduced. In fact, it is the 16:0/18:2 species that is the most decreased. In phosphatidylinositol (PI, **Fig. 4C**), which has a simpler molecular species distribution, the mutation allows the 18:0/18:2 and 18/0/18:3 species to accumulate, while the 16:0/18:2 one is reduced, as in PE. PG is synthesized both by the prokaryotic pathway in the chloroplast and the eukaryotic pathway in the endoplasmic reticulum. For this lipid (**Fig. 4D**) the two 18:0-containing species are also more present in the mutant (ca. 5%) than in WT (less than 1%). Nandi et al.[16] reported an increase in 34:3 PG in the *ssi2* mutant, that we can attribute solely to 16:0/18:3 species, the amount of 16:1/18:2 being unaffected. These authors have not shown the presence of the two 18:0-containing molecules as they did not check PG with two C18 fatty acid chains. Indeed our results with *ssi2* PG clearly establish that PG with two C18 fatty acids exist in plants.

This method of lipid analysis is a powerful tool for studying changes in composition following stresses. For instance, an overnight cold stress (4°C) or dark exposure applied to *Arabidopsis* plantlets induces an increase in 18:3 content that is mainly associated to 16:3/18:3 species in MGDG and 18:3/18:3 ones in DGDG (not shown). Also, the total content in a given phospholipid mass can be almost unchanged while the mass corresponds to two species that vary in an opposite way. It is the case of 36:4-PC in the above situation in which 18:1/18:3 decreases and 18:2/18:2 increases during the stress.

Our examples from situations already known to impact lipid desaturation (desaturase mutant or different species) clearly illustrate that, due to the accuracy in determination of each molecular species present in the various lipid classes, obtained in a single run, mass spectrometry in MRM mode will be useful to follow the lipid changes that accompany the responses of plants to abiotic or biotic stresses.

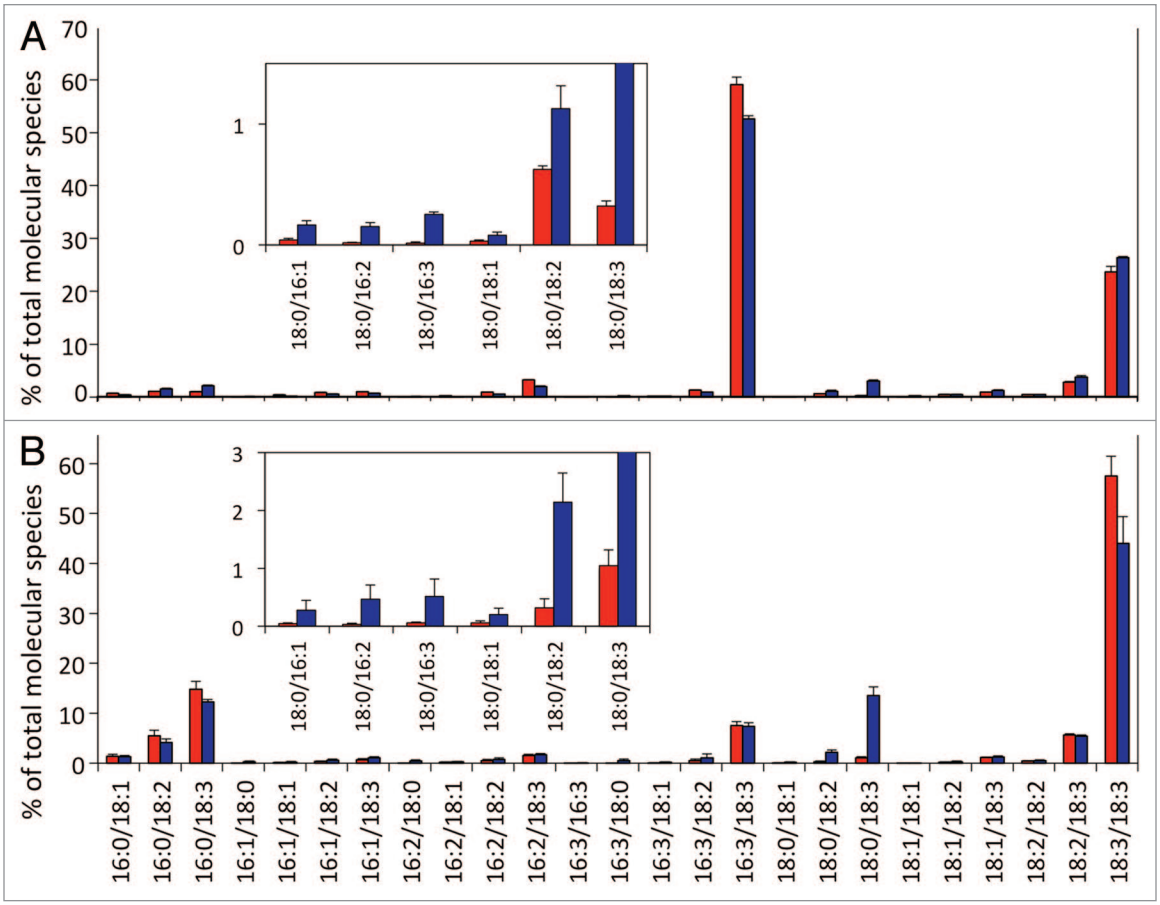

Figure 3. Molecular species composition of MGDG (**A**) and DGDG (**B**) extracted from *Arabidopsis thaliana* cv Nössen leaves. Wild type: red bars. *ssi2* mutant: blue bars. Insert graphs give details of 18:0-containing molecular species. Values are means of 3 independent experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
http://www.landesbioscience.com/journals/psb/article/24118

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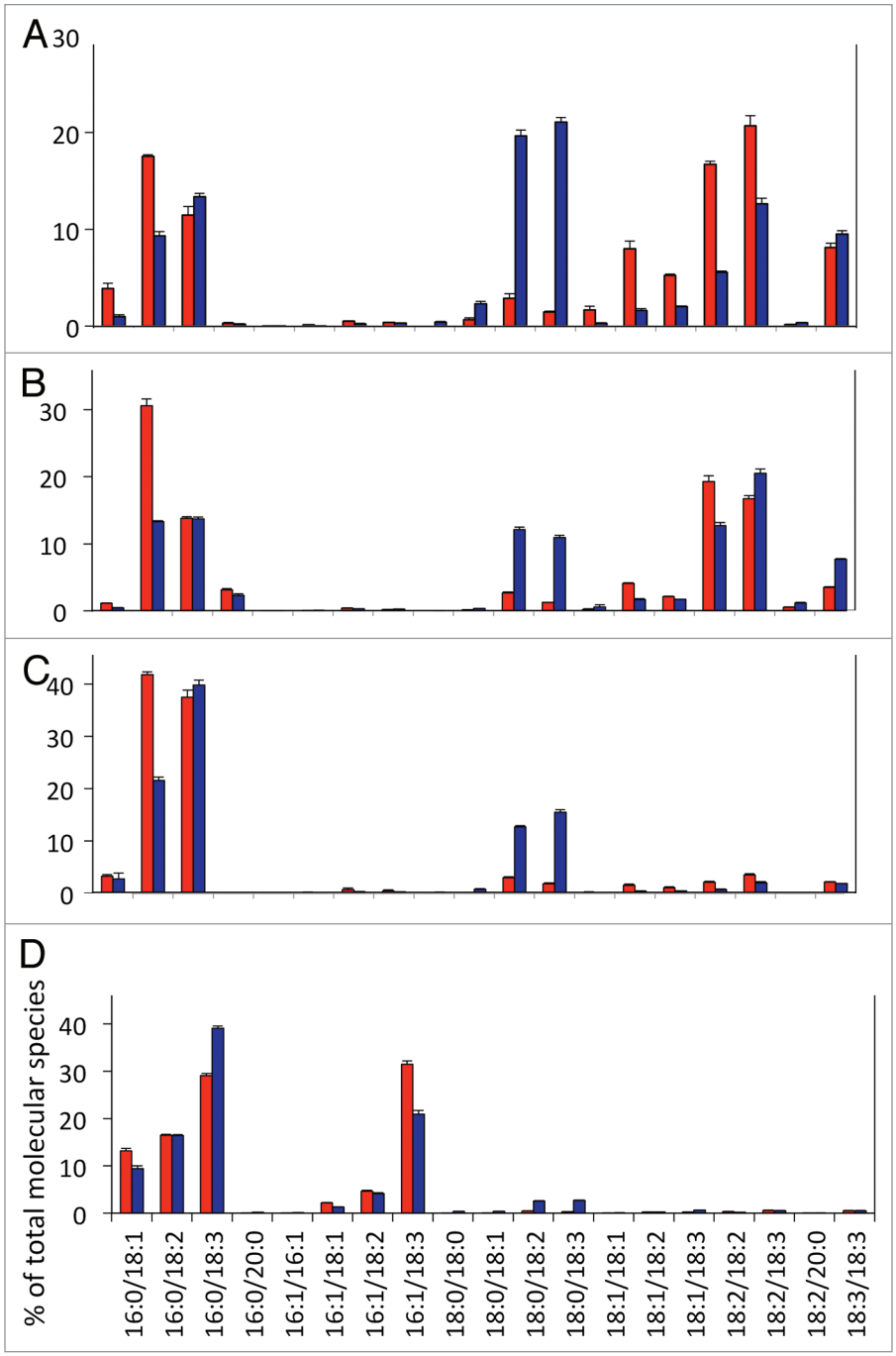

Figure 4. Molecular species composition of PC (**A**), PE (**B**), PI (**C**) and PG (**D**) extracted from *Arabidopsis thaliana* cv Nössen leaves. Wild-type, red bars; *ssi2* mutant, blue bars. Values are means of 3 independent experiments.

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